Preparation and Use of Monoclonal Antibodies to Detect
Phytophthora nicotianae var. *nicotianae**

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Abstract

PEKÁROVÁ-KYNĚROVÁ B., KUTÍKOVÁ M. (1999): **Preparation and use of monoclonal antibodies to detect** *Phytophthora nicotianae* var. *nicotianae*. Pl. Protect. Sci., **35**: 41–46.

A monoclonal antibody (MAb 18) was prepared against purified mycelial proteins from *Phytophthora nicotianae* var. *nicotianae*. The specificity of MAb 18 (IgG class) was tested using indirect ELISA (PTA-ELISA). It cross-reacted with *Phytophthora cactorum, P. cinnamomi, P. cryptogea, P. fragariae*) but not with other fungi (*Fusarium oxysporum, Pythium ultimum* and *P. oligandrum*) and bacteria (*Clavibacter michiganensis* subsp. *michiganensis*) isolated from tomato. *Phytophthora nicotianae* var. *nicotianae* was detected in roots and basal stems of artificially infected young tomato plants using indirect ELISA and immunoprinting.

Key words: *Phytophthora nicotianae* var. *nicotianae*; monoclonal antibodies; indirect ELISA; immunoprinting; tomato

Since the introduction of hybridoma technology by KÖHLER and MILSTAIN (1975), genus- and species-specific monoclonal antibodies (MAbs) have been used successfully in immunoassays for the detection and tracking of fungi in plants and soils (DEWEY 1996). The main advantage of MAbs is that they can be produced in unlimited quantities and preserved indefinitely. In most cases they are highly specific, sensitive and do not cross-react with related and unrelated fungi and extracts from host tissues (DEWEY 1996). These features make them an important tool in modern diagnosis of phytopathogenic fungi.

Our aims were:

1) to prepare MAbs suitable for the detection of *Phytophthora nicotianae* van Breda de Haan var. *nicotianae* (*PNN*)
2) to test these MAbs for the diagnosis of *PNN* on tomato plants.

Based on our experience from previous experiments on the preparation and utilisation of polyclonal antibodies to detect *PNN*, we describe here the preparation and utilisation of MAbs directed against this pathogen.

MATERIAL AND METHODS

Pathogen Material

A virulent isolate of *Phytophthora nicotianae* var. *nicotianae* and isolates of other *Phytophthora* species (*P. cactorum* [Lebert & Cohn] Schroeter, *P. cinnamomi* Rands, *P. cryptogea* Pethybride & Lafferty, and *P. fragariae* Hickman) were obtained from the Collection of Phytopathogenic Microorganisms of the Research Institute of Crop Production, Prague, Czech Republic. The isolates were maintained on solid potato-dextrose medium in 9 cm Petri dishes at 24–25 °C. Hohl's medium (230 ml) in Roux's flasks was used for liquid growth cultures (RIBEIRO 1978). After 21–24 days the cultures were used for antigen preparations.

Antigen Preparation

Purified protein solutions prepared according to KRÁTKÁ *et al.* (1995) were used as immunogens or as antigens in immunochemical tests. The content of proteins was determined according to LOWRY *et al.* (1951).

Preparation of Monoclonal Antibodies

Female Balb/C mice, 6 to 20 weeks old, were given five intraperitoneal injections of approx. 150 µg of proteins at two week intervals. The immunogen was emulsified for the first injection with Freund's complete adjuvant, and with Freund's incomplete adjuvant for subsequent injections. Three days after the sixth injection containing 300 µg of proteins without adjuvant, the mice splenocytes were fused with Sp 2/0 myeloma cells using 50% polyethylene glycol 1 300–1 600 (Sigma).

Growing hybridomas were tested for anti-*P. nicotianae* var. *nicotianae* antibody production using indirect ELISA

on Terasaki microtitre plates. Clones were obtained from hybridoma colonies that grew on soft agar. Each positive cell line was cloned three times. The stable clones were cultured in spinner flasks to 0.5 l for large-scale production of MAbs.

The antibody-containing cell culture supernatants were concentrated by polyethylene glycol approximately 20 times to 25 ml. Antibodies (class IgG) were precipitated with ammonium sulphate and purified subsequently by ion-exchange chromatography on DEAE-matrix. Final concentration of anti-*PNN* IgG was 1 mg/ml. The antibodies were frozen and stored at –20 °C.

Plant Material

For each experiment, fifty young tomato plants (*Lycopersicon esculentum*) of the susceptible cv. Stupické were inoculated with a suspension of *PNN* as described previously (KYNĚROVÁ *et al.* 1998). Mycelial mats were homogenized in 300 ml of sterile distilled water (approx. 10^6 propagules per ml). The plants were watered with this suspension (150 ml of suspension per 50 plants). Twenty-five control plants were watered with sterile distilled water. Three such experiments were performed from April to May.

Roots and basal stems extracts in PBS for ELISA were prepared from 5 to 10 days after inoculation as outlined previously (KYNĚROVÁ *et al.* 1998).

PTA-ELISA Procedure

PTA-ELISA was performed in polystyrene 96-microtitre plates (GAMA) and was used for tests of IgG specificity and for detection of *PNN* on tomato. Wells were coated with antigen diluted in bicarbonate buffer pH 9.6 and incubated overnight at 4 °C (100 μl per well). The plates were washed once with washing buffer (PBS-Tween 20), blocked with 200 μl per well of 1% BSA in washing buffer for 30 min at 37 °C, washed again three times with washing buffer and then incubated with anti-*PNN* IgG diluted in the washing buffer with 1% BSA for 3 h at 37 °C (100 μl per well). After subsequent washing, the sheep anti-mouse IgG coupled with alkaline phosphatase (Boehringer Mannheim GmbH) diluted to 250 mU/ml in 0.05M Tris-HCl with 0.2% BSA was added (100 μl per well) and the plates were incubated for 3 h at 37 °C. The plates were washed, 100 μl of p-nitrophenylphosphate (1mg/1 ml) diluted in diethanolamine buffer (pH 9.8) was added to each well, and after 60 min incubation the absorbances at 405 nm were read with an automatic reader (E-max, Molecular Devices). The mean absorbance was calculated after substracting the absorbance given by the substrate in wells not coated with antigens.

Immunoprinting Procedure

Immunoprinting was used to detect *PNN* in tissue parts of tomato plants. Cuts of healthy and infected plants were printed on nitrocellulose membranes (Millipore Corpora-

tion, 0,45 μm of porosity). The membranes were immersed in PBS pH 7.2 containing 5% skimmed milk for 1 h at room temperature. They were next incubated with anti-*PNN* IgG diluted to 1 μg/ml in PBS with 5% skimmed milk for 1 h at room temperature. After three rinses with PBS and one rinse with TBS (0.01M Tris, 0.015M NaCl, pH 8.1) the membranes were incubated with sheep anti-mouse IgG coupled with alkaline phosphatase (Boehringer Mannheim GmbH) diluted to 250 mU/ml in TBS with 5% skimmed milk for 1 h at room temperature. After three rinses with TBS and one rinse with TBS-$MgCl_2$ (0.1M Tris, 0.15M NaCl, 0.005M $MgCl_2 \times 6\ H_2O$, pH 9.1) the membranes were immersed in the BCIP/NBT substrate solution (Sigma). The colour reactions were stopped by three rinses in distilled water. The membranes were dried and evaluated. All incubation steps were performed during orbital shaking (Minishaker IKA, 300 rpm).

Image Analysis of Prints

Prints on the nitrocellulose membranes were first digitized using a scanner, then transformed into a 256 gray scale where '0' represents black and '255' white colour. The intensity of prints was subsequently analysed using SIGMASCAN by drawing an 'intensity line' through every print. Differences between the intensity values of the prints were subsequently evaluated by ANOVA.

RESULTS

Preparation and Test of Antibodies

In one fusion 15 positive hybridomas were identified by indirect ELISA on Terasaki plates, but only one was recovered in a clonal form (MAb 18). Fig. 1 shows data obtained with *PNN* antigen (used as immunogen) coating 0.1–50 μg protein/ml and purified antibodies at a concentration of 1μg/ml in indirect ELISA. The absorbance obtained varied between experiments, as is apparent from
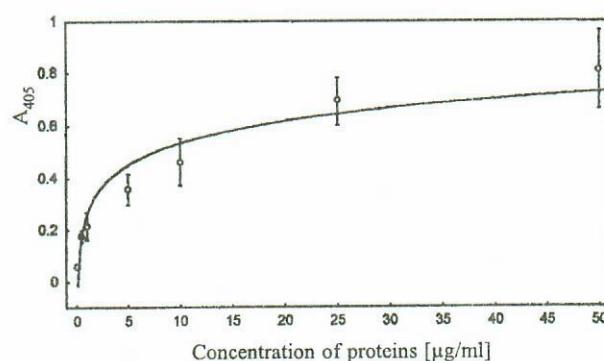

Fig. 1. Logarithmic model fitted to the relationship between concentration of proteins and absorbance obtained in the detection of *P. nicotianae* var. *nicotianae* antigens by indirect ELISA. Wells were coated with antigens at concentrations ranging from 0.1 to 50 μg fungal protein/ml. MAbs were added at a concentration of 1 μg/ml. Bars represent standard errors of means from ten separate assays, each in three replications
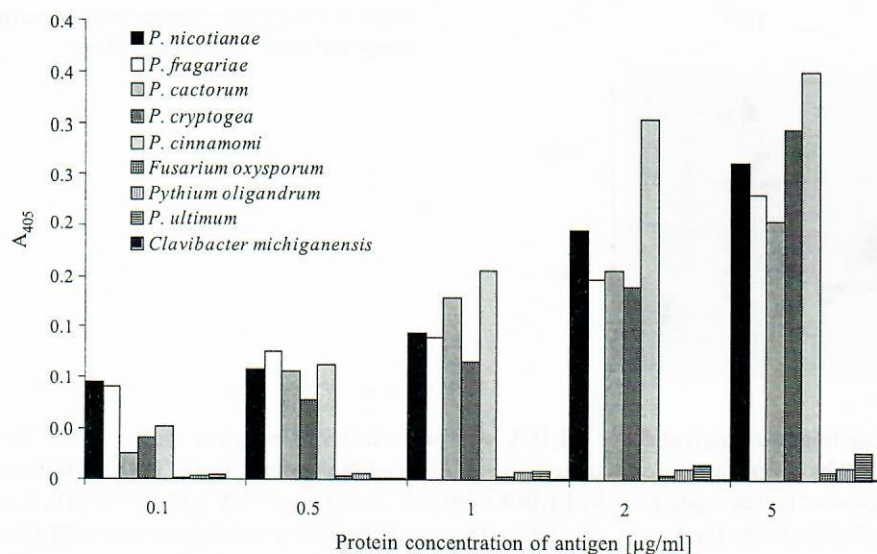
Fig. 2. Indirect ELISA: cross-reactions of other tested fungi

the standard error whiskers. To these data a model was fitted. The best fit was obtained by a logarithmic model, specifically $y = 0.259 + 0.275 * \log(x)$.

MAb 18 reacted strongly with all tested species of the genus *Phytophthora*. The differences were quantitative (Fig. 2). MAb 18 did not recognize constituents of other fungi and bacteria that often occur in tomato plants such as *Fusarium oxysporum*, *Pythium ultimum*, *P. oligandrum* and *Clavibacter michiganensis* subsp. *michiganensis*.

Detection of *PNN* on Tomato Plants

Five categories of plants were analyzed: (1) healthy, uninoculated plants = control; (2) infected plants without visible symptoms = degree of disease severity 0 (DS0); (3) infected plants with mild wilting of leaves = degree of disease severity 1 (DS1); (4) infected plants with mild wilting of leaves and mild stem shrinking = degree of disease severity 2 (DS2); (5) infected plants with severe wilting of leaves and severe stem shrinking = degree of disease severity 3 (DS3). The presence of the pathogen was eval-

Fig. 3. ELISA values (mean ± SE) obtained from healthy and artificially infected tomato extracts with *P. nicotianae* var. *nicotianae* at three levels of infection. Control: healthy plants inoculated with water. DS1: plants with mild wilting of leaves. DS2: plants with mild wilting of leaves and mild stem shrinking. DS3: plants with severe wilting of leaves and severe stem shrinking

uated by means of indirect ELISA (Fig. 3) and immunoprinting (Fig. 4).

The absorbance values obtained by ELISA (Fig. 3) were significantly higher in root samples with DS1, DS2 and DS3 than in the control set ($P < 0.01$, Kruskal-Wallis ANOVA). In basal stem samples only the absorbance values of samples with DS1 and DS2 were higher than the control ($P < 0.05$); the absorbances of DS3 were lower than those of DS1 and DS2 in both roots and stems. Absorbance values greater than 0.25 for root samples and > 0.15 for stem samples identify infected tissue.

The intensity values of stem and root prints are shown in Fig. 5. The lightest are the prints of the control, the darkest are samples with DS2 and DS3. The prints of stems were in all cases significantly lighter ($P < 0.01$) than those of roots, except samples with DS3 ($P = 0.28$). On prints of stems the intensity increased (became darker) with the level of infection. On root prints there was no difference between infected plants rated DS1, DS2 and DS3 ($P > 0.42$), but all were significantly darker than the control prints ($P < 0.05$).

MAb 18 used in ELISA and immunoprinting was not sufficiently sensitive to detect infection in samples rated DS0 (not shown here).
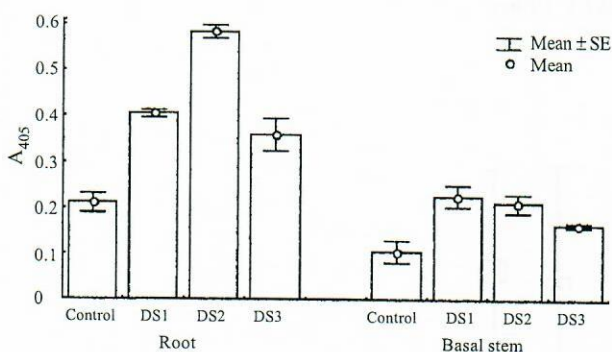
DISCUSSION

Our results suggest that the specificity of MAb 18 raised to *PNN* is limited by the genus of the pathogen. This also applies to polyclonal antibodies directed against *PNN* (KRÁTKÁ *et al.* 1995; KYNĚROVÁ *et al.* 1998). Thus, monoclonal antibodies are not necessarily more specific than polyclonal ones. It can be assumed that the specificity depends largely on the presence of common and characteristic antigenic determinants in the proteins. MERZ *et al.* (1969) and HALSALL (1976) found that *Phytophthora* species contain common antigens. In general, it is much easier to raise specific MAbs to saprophytic and ne-
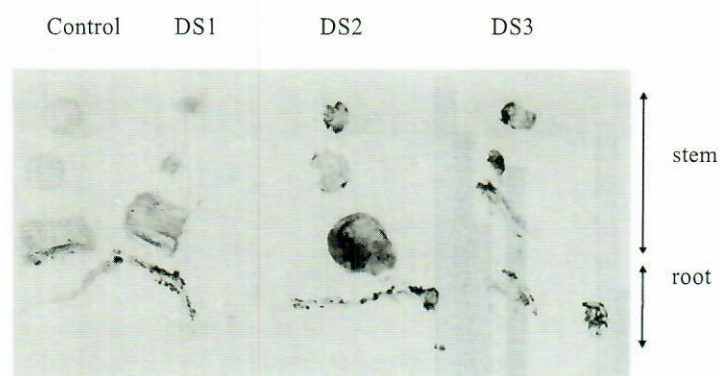
Fig. 4. Nitrocellulose surface with prints of stems and roots from tomato plants

crotrophic fungi than to specialized pathogenic fungi, and cell lines secreting genus-specific MAbs occur far more frequently than those secreting species- or isolate-specific MAbs (DEWEY 1996). This is probably due to the fact that the former secrete greater quantities of proteins and glycoproteins, particularly hydrolytic enzymes, than do pathogenic fungi (DEWEY 1998).

Two immunological techniques, PTA-ELISA and immunoprinting, were used to detect the pathogen on tomato using prepared MAb 18. Both techniques were not sensitive enough to detect early and latent infection (DSO), i.e., before symptoms could be seen. The problem may be related to host compounds that interfere either with the binding of the fungal antigen to the surface of microtitre wells and nitrocellulose or with the antigen–antibody reaction (AVILA *et al.* 1995; DEWEY 1996).

Detection of the pathogen was possible in roots and basal stems of young tomato plants 5 days after inoculation when the first visible symptoms appeared. Both techniques were sufficiently suitable to detect this infection level. However, the sensitivity of ELISA was low if the substrate *p*-nitrophenolphosphate was used. The sensitivity could be improved considerably by application of the GIBCO enzyme amplification system (RABENSTEIN & SCHLIEPHAKE 1996). Using this method, RABENSTEIN *et al.* (1998) found a good correlation between visual scoring and ELISA values for detection of *Drechslera teres* by MAbs.

ELISA was not sensitive enough to detect DS3. The same was experienced in the detection of *PNN* on tomato by ELISA with polyclonal anti-*PNN* IgG (KYNĚROVÁ *et al.* 1998). This could be due to inhibition of the ELISA reaction by oxidized polyphenols which were frequently shown to be produced in severely diseased plants (WERRES 1988; GABLER & URBAN 1995).

Setting positive/negative thresholds in the detection of fungal pathogens in plants is not easy in ELISA. According to CONVERSE and MARTIN (1990) there is no absolute threshold value in ELISA to differentiate between the reliable detection of the fungus and background reactions. ELISA values might be influenced by the way the fungal antigen is released from infected plant tissues, dilutions of antigens, the host-pathogen system, inhibition of ELISA by host and pathogen metabolites etc. To avoid problems associated with the difficulty to set thresholds when determining healthy or infected states, we always compared reactions of infected material and the control. In the system *Phytophthora*–tomato, positive absorbance values had to be greater than 0.25 for roots and 0.15 for stems. The positive samples should be verified by other diagnostic tests. According to our experiences, it is necessary to define conditions and methods for each host–pathogen system when determining positive/negative thresholds. Similar conclusions were reached by other authors (DEWEY 1996).
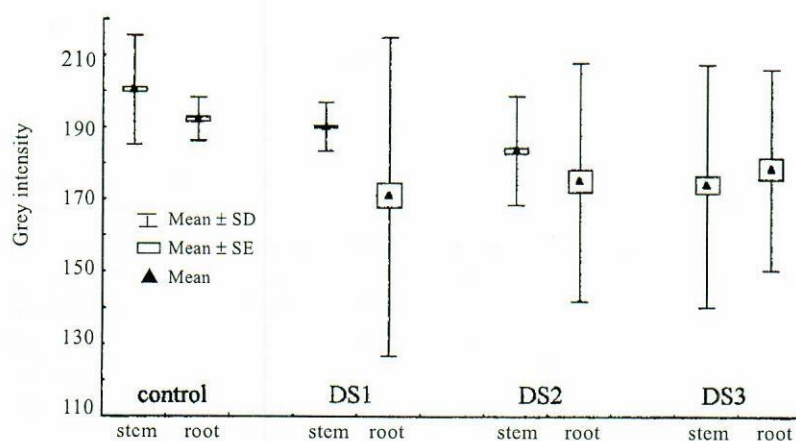

Fig. 5. Comparison of the intensity of stem and root prints from tomato (control, DS1, DS2, and DS3) on a 256 gray scale: '0' represents black and '255' white colour

In contrast to ELISA, there was a strong reaction of MAb 18 also with samples with DS3 when tested by immunoprinting. This may stem from the advantage that immunoprinting does not require extract preparation and a possible modification of epitopes during that process.

Each detection test is a compromise between sensitivity and specificity. It is very difficult to devise tests in which both sensitivity and specificity are high. Depending on the material, nature of the pathogen, and consumer interests the producer has to make a choice which kind of test to use (HUTTINGA 1997).

Production of more specific new MAbs still seems to be necessary. This might be achieved by purified antigen preparations or by using sap from infwcted leaves as antigens (DEWEY 1998).

Acknowledgements

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Souhrn

PEKÁROVÁ-KYNĚROVÁ B., KUTÍKOVÁ M. (1999): **Příprava a použití monoklonálních protilátek k detekci** *Phytophthora nicotianae* var. *nicotianae*. Pl. Protect. Sci., **35**: 41–46.

Pro detekci *Phytophthora nicotianae* var. *nicotianae* (*PNN*) byla připravena monoklonální protilátka (MAb 18). K imunizaci laboratorních myší byl použit purifikovaný extrakt z myceliální hmoty jmenované houby. Nepřímou metodou ELISA (PTA--ELISA) byla testována specifičnost protilátky (třída IgG). Protilátka křížově reagovala i s jinými testovanými druhy rodu *Phytophthora* (*P. cactorum*, *P. cinnamomi*, *P. cryptogea*, *P. fragariae*), avšak nereagovala s dalšími patogeny izolovanými z rajčete

(*Fusarium oxysporum, Pythium ultimum, P. oligandrum* and *Clavibacter michiganensis* subsp. *michiganensis*). *PNN* byla detekována metodou ELISA a imunoprintem v kořenech a bazálních částech mladých rostlin rajčete, které byly uměle inokulovány tímto patogenem.

Klíčová slova: *Phytophthora nicotianae* var. *nicotianae*; monoklonální protilátky; nepřímá ELISA; imunoprint; rajče

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